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Genotyping and detection of multiple infections of *Toxoplasma gondii* using Pyrosequencing

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Abstract

A Pyrosequencing™ assay, based on *SAG2* gene polymorphisms, was designed for genotyping and detection of multiple infections of *Toxoplasma gondii*. The assay was tested on samples spiked with DNA from single and multiple genotypes of *T. gondii* and also on a DNA sample from the brain of a rat with multiple infections. To evaluate the comparative efficacy of the assay, identical samples were also analysed by PCR-restriction fragment length polymorphism (RFLP) and dideoxy sequencing. The Pyrosequencing assay was found to be superior to the two conventional techniques. Genotyping and detection of multiple alleles were possible after a single PCR assay in duplex format, from both the spiked and direct samples. The simplex PCR assay enabled accurate quantification of the different alleles in the mix. In comparison, PCR-RFLP and dideoxy sequencing were neither able to unequivocally detect multiple genotype infections, nor quantify the relative concentrations of the alleles. We conclude that Pyrosequencing offers a simple, rapid and efficient means for diagnosis and genotyping of *T. gondii*, as well as detection and quantification of multiple genotype infections of *T. gondii*.

Keywords: *Toxoplasma gondii*; Genotyping; *SAG2*; Multiple infection; Pyrosequencing; PCR-RFLP; Dideoxy sequencing

1. Introduction

Toxoplasma gondii is a protozoan parasite of medical and veterinary importance, which can infect almost all warm-blooded animals (Dubey and Beattie, 1988). The manifestation of the disease ranges from chronic infection in most animals, to acute disease and death in some. The parasite also causes economic losses due to foetal death and abortion, especially in sheep. Toxoplasmosis is a zoonotic disease and has been documented as an important food-borne infection in humans (Jones et al., 2001). It is also associated with complications and death in immunocompromised patients (Tenter et al., 2000).

In recent years, there has been a greater emphasis on genetic analysis of isolates of *T. gondii* and their importance in disease, as there is increasing evidence on the relationship between strain type and disease outcome, especially in humans (Boothroyd and Grigg, 2002). Howe and Sibley (1995) determined that the *T. gondii* isolates could be grouped into three genotypes, Types I, II and III. A PCR-restriction fragment length polymorphism (RFLP) assay was designed to easily differentiate the three genotypes (Howe et al., 1997), based on polymorphisms in the *SAG2* gene. Subsequently, numerous high-resolution assays have been developed for typing *T. gondii*, ranging from microsatellite analysis (Blackston et al., 2001; Ajzenberg et al., 2002), to the use of synthetic peptides (Kong et al., 2003). While these high-resolution strain-typing techniques provide superior genetic data, they are more labor intensive and the PCR-RFLP based genotyping (Howe et al., 1997) is still used for the preliminary genotyping of isolates.

Most of the isolations of *T. gondii*, from domestic and wild animals, comprise of a single genotype. The presence of multiple genotypes have been reported, based on the data from dideoxy sequencing of the polymorphic regions of

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the *SAG*2 gene of *T. gondii* from commercial meat products (Aspinall et al., 2002) and based on microsatellite analysis (Ajzenberg et al., 2002). Subsequently, multiple genotypes have been isolated from a single host by mouse bioassay (Dubey et al., 2003). However, it is unclear if the low incidence of infection with multiple *T. gondii* genotypes reflects the true state of affairs or is due to difficulty in effective diagnosis (Villena et al., 2004). Currently, sequencing, microsatellite analysis and to a lesser extent, improper or partial digestion during PCR-RFLP, are used to determine mixed infections. However, there is no simple and specific assay for unequivocal detection of multiple genotype infections of *T. gondii*.

Pyrosequencing (Ronaghi et al., 1998) is a technology ideal for detecting single nucleotide polymorphisms (SNPs) and short-read sequencing. It is an accurate, simple and flexible bioluminometric method, which does not need labeled nucleotides or gel electrophoresis. In this technique, an enzymatic cascade reaction is used to convert the inorganic pyrophosphate (PPi) released during the incorporation of deoxynucleotide triphosphate, into proportional amounts of visible light, which can then be measured. The technique has great application, especially for the detection of SNPs and is being increasingly used for genotyping pathogens, including bacteria (Nilsson et al., 2004), viruses (Elahi et al., 2003) and metazoan parasites (Troell et al., 2003).

In this paper, we describe the usefulness of a Pyrosequencing assay to determine the genotypes of *T. gondii* and to detect and quantify multiple genotype infections. We also compare this technique with the conventionally used assays, PCR-RFLP and dideoxy sequencing.

2. Materials and methods

2.1. Parasites

Three *T. gondii* isolates, TgCat Br-30 (Type I, Dubey et al., 2004), ME 49 (Type II, Howe and Sibley, 1995) and VEG strain (Type III, Dubey et al., 1996), were used in the study. The isolates were available as sporulated oocysts. The oocysts were fed to outbred Swiss Webster mice and the parasites were transferred to CV1 cell culture from the mesenteric lymph node extracts of mice. Upon complete destruction of the monolayers, the parasites were collected, DNA extracted using DNAeasy tissue kit (Qiagen, Valencia, CA) and quantified. The final DNA working solutions (in Tris buffer) were adjusted to deliver approximately 50 ng of DNA per μl. The experiments in animals were performed according to United States Department of Agriculture's approved guidelines for animal care.

2.2. Genotyping

The comparative efficacy of Pyrosequencing, PCR-RFLP and dideoxy sequencing techniques, to determine the genotypes and diagnose mixed infection, was tested. Eight sets of reactions were run using samples spiked with known quantities of DNA of either single genotype or multiple genotypes (Table 1). Additionally, DNA extracted from the brain of a rat that contained all three genotypes, was also included. This rat had previously been infected simultaneously with 1000 oocysts each of all three genotypes. The tissue cysts from brain were individually picked (Sreekumar et al., 2003) and genotyped by PCR–RFLP and all three genotypes were found to be present (Table 1). The DNA was extracted as above and used for PCR. All reactions were performed three times to ensure repeatability.

2.2.1. Pyrosequencing

Pyrosequencing Assay Design Software (ADSW) was used to design assays to determine the SNPs at the recognition sites for enzymes *Sau*3A1 (for Type III) and *Hha*1 (for Type II). The assay was designed using the GenBank sequences of the 5′ and 3′ termini of *SAG2* gene of Type I (M33572), Type II (AF249696, AF249697), and Type III (AF249798, DQ000461). The primers PyroT3F (5′-TGC TGC AGT GAC CCA TC-3′) and PyroT3BioR (5′-Biotin- CTA GAA CTG CAA CCC GTG AAA-3′) were used to amplify the region of *SAG2* gene containing the polymorphic *Sau*3A1 recognition sequence. The primer PyroT3Seq (5′-GAG CGC TGC TTG CGA T-3′) was used as the sequencing primer. The region of *SAG2* containing the *Hha*1 recognition sequence was amplified with the primers PyroT2BioF (5′- Biotin-CGG ATC TGC GAT TAT GTG ACA-3′) and PyroT2R (5′- CGG AAC ACT GGT TGT GTC TG-3′), while PyroT2Seq (5′- GTC TGG CGG AAA AGC-3′) was used as sequencing primer. The following thermal cycling conditions were used: after an initial denaturation of 94 °C for 5 min, 10 cycles at 68 °C (94 °C/20 s, 68 °C/30 s, 72 °C/30 s), 10 cycles at 65 °C (94 °C/20 s, 65 °C/30 s, 72 °C/30 s), and 15 cycles at 60 °C (94 °C/20 s, 60 °C/30 s, 72 °C/30 s) were used for amplification, followed by a final extension at 72 °C for 10 min.

Assays were run in simplex (any one set of primers) and duplex (both sets of primers) formats and the PCR products were analysed on the PSQ96 HS system (Biotage AB). Samples were prepared for analysis as recommended by the manufacturer (Biotage AB; Dunker et al., 2003). The biotinylated PCR products were immobilized to streptavidin coated sepharose beads (Streptavidin Sepharose™ HP, Amersham Biosciences Ltd) in 1×binding buffer. After immobilization, the fluid was removed by aspirating the beads with Vacuum Prep Tool (Biotage AB) and the beads were treated for approximately 5 s with 70% ethanol, 5 s with denaturation buffer, and 5 s with washing buffer. Finally, the beads were released into Pyrosequencing reaction plates containing 12 μl annealing

Table 1
Comparative efficacies of PCR-RFLP, dideoxy sequencing and Pyrosequencing assays in genotyping and detection of multiple genotype infections of *Toxoplasma gondii*

Sample No.	Genotype(s) (ng DNA per reaction)	Allele detection (A) and quantification in percentage (B) by					
		PCR-RFLP		Sequencing		Pyrosequencing	
		A	B	A	B	A	B
1	I (100)	Yes	100	Yes	100	Yes	100
2	II (100)	Yes	100	Yes	100	Yes	100
3	III (100)	Yes	No[a]	Yes	100	Yes	100
4	I (50) and II (50)	Yes	No	Yes[b]	No	Yes	44.5(I), 55.5 (II)
5	I (50) and III (50)	Yes	No	Yes[b]	No	Yes	37.6(I), 62.4 (III)
6	II (50) and III (50)	Yes	No	Yes	No	Yes	40.6(II), 59.3(III)
7	I (50), II (50) and III (50)	Yes	No	Yes[c]	No	Yes	27.5(I), 31.4(II), 41.1(III)
8	I (10) and III (90)	No[d]	No	No	No	Yes	9.3 (I), 90.7(III)
9	I, II and III (100)[e]	Yes	No	No	No	Yes	No

[a] Incomplete digestion, falsely indicating more than one allele.
[b] Numerous small spurious peaks present in the sequence chromatograms.
[c] The secondary peaks at the *Hha*1 site are very weak and masked by the stronger primary peak, falsely indicating the presence of just one allele.
[d] The prominent undigested band in PCR-RFLP appeared to be due to improper digestion rather than the presence of multiple alleles.
[e] Contained 12% Type I, 20% Type II and 68% Type III tissue cysts.

buffer with 0.5 μM of each sequencing primer. Primer annealing was performed at 90 °C for 2 min. The reactions were allowed to cool to room temperature.

The samples were analyzed on a PSQ HS 96A instrument, using SNP reagents (Biotage, AB) according to standard protocols. The order of nucleotide dispensations was determined by the PSQ HS 96 SNP software (Biotage AB), which was also used for automatic assay evaluation, genotype scoring, and allele quantification analyses.

The samples were initially analysed using the duplex PCR assay. The simplex PCR assays were then used to validate the duplex assay and quantify the different alleles in the DNA mix.

Data on primer design and theoretical histograms of simplex and duplex PCR assays are depicted as Supplementary data.

2.2.2. PCR-RFLP

PCR-RFLP at the *SAG2* loci was performed according to Howe et al. (1997) with minor modifications. A new internal reverse primer (5′-AAT TCT GAG AGA TCC AGC-3′) was designed for the 3′ end for determining Type II isolates. PCR reactions were run in 25 μl volumes, each with approximately 100 ng of DNA. 10 μl of the PCR product was digested with 10 units of the appropriate enzymes (*Sau*3A1 for Type III and *Hha*1 for Type II) and the digestion products were electrophoresed on a 2% gel. The presence of digestion or the lack of it at the specific sites was documented.

2.2.3. Dideoxy sequencing

The PCR products generated from the above reactions were also sequenced. Fifteen μl of the nested product was electrophoresed in 2% gels. The specific amplicons were purified from agarose gel and directly sequenced in both directions using the Big Dye terminator system, version 3.1 (Applied Biosystems, Foster City, CA) using an ABI 373× 1 capillary sequencer. The sequence chromatograms were edited using SEQUENCHER 4.1 software (Genecodes Corp., Ann Arbor, MI). The regions of the chromatograms representing the polymorphic nucleotides at the enzyme recognition sites were examined for the presence or absence of double peaks to identify mixed infection.

3. Results

3.1. Pyrosequencing

The target fragments of 77 bp (Type III) and 88 bp (Type II) could be amplified efficiently using both the simplex and duplex formats of PCR. The duplex analysis clearly identified the three different genotypes. It also detected the presence of mixed genotypes in the spiked samples as well as the direct sample from the rat brain (Supplementary data).

The pyrograms (from *Sau*3A1 and *Hha*1 recognition sequences) generated by the simplex PCR reactions are depicted in Figs. 1 and 2. The peaks highlighted by the gray boxes are the informative peaks in the assays. Results from analyses using the allele quantification software function are given as the % concentration of the different alleles in the samples. The pyrogram pairs for samples 1 through 3 unequivocally differentiated the three different genotypes. The peaks at both restriction sites for these three samples are present at 100%, clearly indicating the presence of single alleles. For samples 4 through 9, the pyrograms detected the presence of more than one allele. They also revealed the relative concentration of the different alleles in the samples. The technique not only detected the presence of Type I and

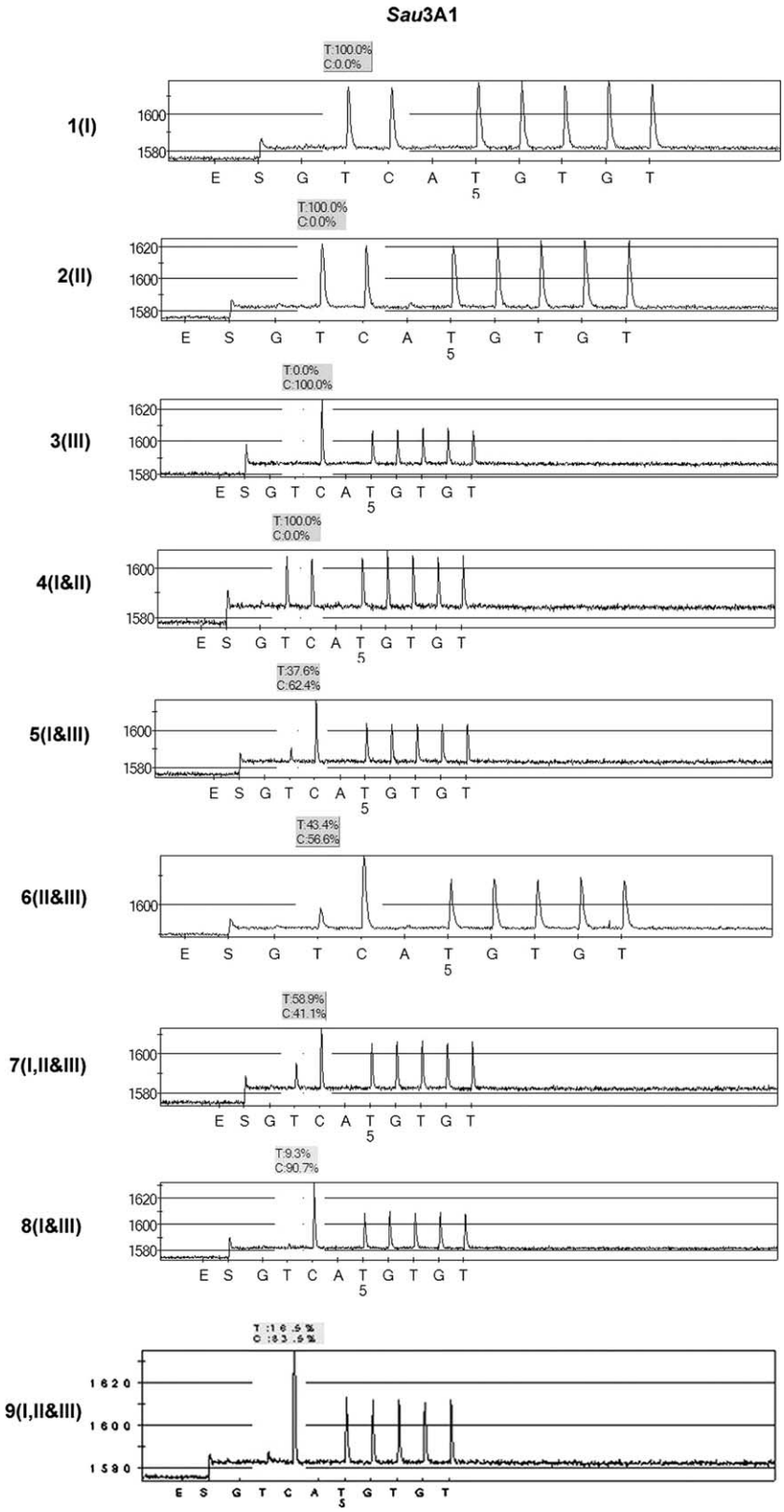

Fig. 1. Results of simplex PCR Pyrosequencing assay detecting SNPs at the *Sau*3A1 site of *SAG2* gene, from samples spiked with single and multiple genotypes of *Toxoplasma gondii* DNA (1–8) and direct sample from the brain of a rat (9). The genotype(s) of the samples are indicated in parenthesis. The peaks in the gray boxes are the informative peaks. The residues 'G' and 'A' flanking the gray box are the negative controls, while the rest of the sequence (TGTGT) acts as the positive control. Samples containing single genotype (1–3) and multiple genotypes (4–9) generate clear, unambiguous signals with reasonably accurate quantification indicated in relative percentages. Refer to Table 1 for the relative concentrations of genotypes in mixed samples.

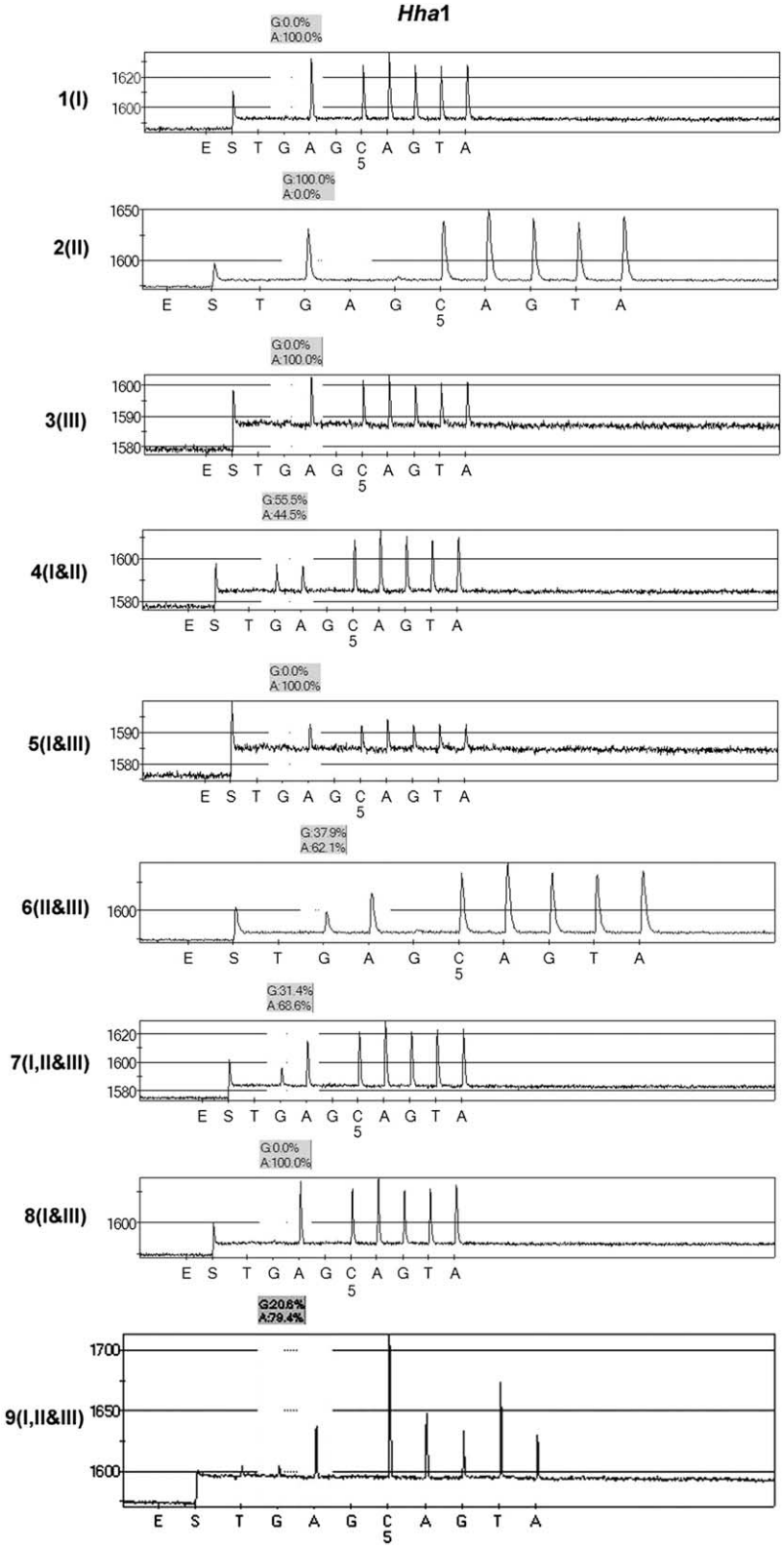

Fig. 2. Results of simplex PCR Pyrosequencing assay detecting SNPs at the *Hha*1 site of *SAG2* gene, from samples spiked with single and multiple genotypes of *Toxoplasma gondii* DNA (1–8) and direct sample from the brain of a rat (9). The genotype(s) of the samples are indicated in parenthesis. The peaks in the gray boxes are the informative peaks. The residues 'T' and 'G' flanking the gray box are the negative controls, while the rest of the sequence (CAGTA) acts as the positive control. Samples containing single genotype (1–3) and multiple genotypes (4–9) generate clear, unambiguous signals with reasonably accurate quantification indicated in relative percentages. Refer to Table 1 for the relative concentrations of genotypes in mixed samples.

Type III alleles in sample 7, but also accurately estimated their relative proportions as 9.3 and 90.7%.

3.2. PCR-RFLP

The restriction patterns of the samples are depicted in Fig. 3. The samples spiked with either Type I (sample 1) or Type II (sample 2) DNA revealed clear RFLP patterns, with lack of digestion at both sites for the former and digestion only at the *Hha* 1 site for the latter (Fig. 3). The RFLP pattern of sample 3 (Type III) showed the presence of digestion at the 5′ end, and lack of digestion at the 3′ end. However, the digestion at the 5′ end was incomplete as evident by the presence of an undigested fragment (double arrow) falsely indicating the presence of more than one allele. The RFLP patterns of all the mixed samples revealed partial digestion at one or both the sites. Thus, partial digestion was noticed at the *Hha*1 site for sample 4 (mix of Type I and II), *Sau*3A1 site for sample 5 (mix of Types I and III) and at both sites for sample 6 (mix of Type II and III). In samples 7 and 9 (mix of all 3 genotypes), the RFLP patterns were similar to sample 6 (Types II and III). However, for sample 7, the digestion products at both sites were almost imperceptible (arrows). The digestion pattern at the *Sau*3A1 site for sample 8 (10-fold Type III to Type I) appeared similar to that of sample 3, with a strong band of digestion products and a faint undigested band.

3.3. Dideoxy sequencing

The sequence chromatograms (Fig. 4) for sample 1 and sample 2 indicated the presence of GATT at the 5′ location for both and GCAC and GCGC at the 3′ location, respectively. However, unlike PCR-RFLP, sequence chromatogram of sample 3 showed the presence of clear GATC and GCAC chromatograms, unequivocally indicating the presence of only Type III. With mixed DNA samples, the presence of double peaks resulted in default read of ‘N’ at

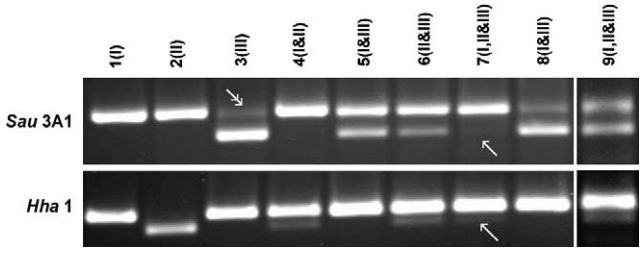

Fig. 3. PCR-RFLP at the 3′ (*Hha*1) and 5′ (*Sau*3A1) termini of the *SAG2* gene from samples spiked with single and multiple genotypes of *Toxoplasma gondii* DNA (1–8) and direct sample from the brain of a rat (9). The genotype(s) of the samples are indicated in parenthesis. Note that the RFLP fingerprint of the sample with Type III DNA (3) has an undigested band (double arrow) erroneously indicating mixed infection. The bands indicating digestion products in lane 7 (mixture of Types, I, II and III) are barely discernible (arrows) giving an appearance that it has only Type I DNA.

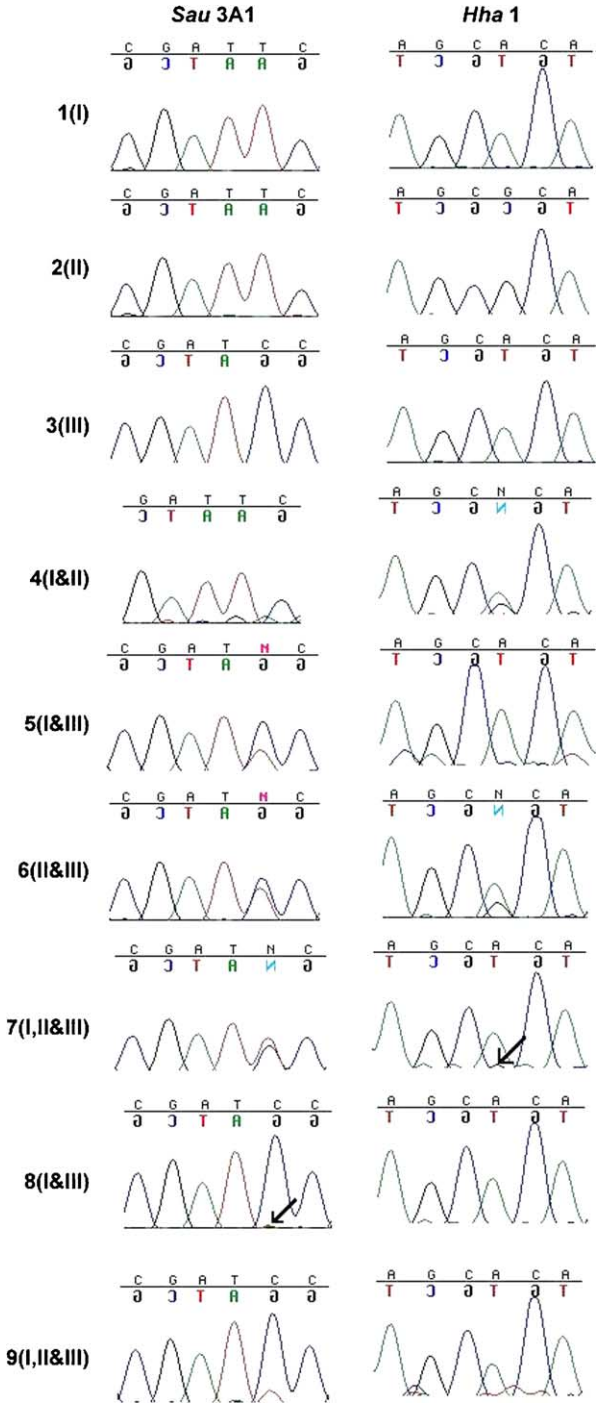

Fig. 4. Sequence chromatograms at the *Sau*3A1 and *Hha*1 enzyme recognition sequences of *SAG2* gene of *Toxoplasma gondii* from samples spiked with single and multiple genotypes of *T. gondii* DNA (1–8) and direct sample from the brain of a rat (9). The genotype(s) of the samples are indicated in parenthesis. Multiple genotypes are indicated by the presence of double peaks at the polymorphic sites leading to a default read of ‘N’ at the *Hha*1 site of sample 4, *Sau*3A1 site of sample 5 and 7 and both sites of sample 6. The secondary peaks are too small (arrows) in samples 7 and 8 to unequivocally diagnose mixed infection. The secondary ‘T’ peak is masked by the primary ‘C’ peak at the *Sau*3A1 site of sample 9. Note the presence of spurious secondary peaks in the chromatograms of samples 4, 5 and 9.

the polymorphic loci of the enzyme recognition sequences. Thus, the presence of 'N' at the *Hha*1 site of sample 4, *Sau*3A1 sites of samples 5 and 7 and both sites of sample 6 indicated mixed infections. The secondary peaks were too insignificant (arrows, Fig. 4) at the *Hha*1 site of sample 7 and *Sau*3A1 site of sample 8, that they were masked by the more dominant peaks, falsely indicating the presence of a single allele. Similarly, the secondary 'T' peak at the *Sau*3A1 site of sample 9, though significantly strong, was still masked by the much stronger 'C' peak, resulting in a default read of 'C'. Spurious peaks were noticed at the *Sau*3A1 sites of sample 4 and *Hha*1 sites of samples 5 and 9.

4. Discussion

The efficacy of Pyrosequencing, PCR-RFLP and dideoxy sequencing techniques were compared in terms of their ability to detect and quantify multiple genotype infections of *T. gondii*.

Pyrosequencing analyses using both simplex and duplex PCR formats provided unequivocal results, identifying the genotypes of single and mixed infections. The samples 4–7 were spiked with approximately the same quantity (50 ng) of the different genotypes. While the quantification of sample 4 (44.5% of Type I and 55.5% of Type II) was as expected, the others were slightly biased in favor of the Type III genotype. All DNA samples were extracted from ripe cultures of tachyzoites in CV1 cells. It is entirely possible that there were relatively more tachyzoites in the culture with Type III tachyzoites, thus contributing more parasite DNA to the final sample. It is also possible that the Type III products were preferentially amplified than the competing allele, thus resulting in more PCR products. Preferential amplification of one allele over the other has been documented (Walsh et al., 1992). Considering the above factors, the results on quantification obtained herein are accurate.

Although Pyrosequencing can clearly detect mixed infections, it can be difficult to distinguish between samples containing mixtures of Type II and Type III from those containing all three. For example, the pyrograms of samples containing Types II and III (sample 6) when compared with those of samples containing all three genotypes (samples 7 and 9) are very similar. This is due to the fact that a combination of signals from two different sites (*Sau*3A1 and *Hha*1) is needed for the analysis. For single infections, the peak patterns of the pyrogram pairs can be used to clearly identify Type I vs. Type II vs. Type III. However, in the case of mixtures, peak pattern alone cannot be used to identify mixtures. In mixed samples, all types will contribute some level of signal at the cytosine residue in the *Sau*3A1 sequence. Likewise, in mixtures containing Type I or Type III, both will contribute some degree of signal to the adenine residue in the *Hha*1 sequence. Allele quantification reports the percentage signal at each of the nucleotides and thus provides a means to estimate the proportion of each of the Types in the mixtures with acceptable accuracy. Sensitivity and variability will depend on the relative amounts of each of the represented types. However, all combinations of only two genotypes can easily be distinguished and quantified. In the case of sample 7, the relative concentration of each allele could be deduced after analyzing the results using the allele quantification functionality of the software. Sample 7 had been spiked with approximately 33% of each allele (Table 1). The quantification results were reasonable (27.5% of Type I, 31.4% of Type II and 41.1% of Type III) with a slight bias towards Type III. Although the presence of multiple genotypes was detected in the sample from rat (sample 9), the relative concentrations of different alleles were given in low confidence due to low signals, which fell below the software cutoff.

In comparison, it can be surmised that Pyrosequencing assay was superior to PCR-RFLP and dideoxy sequence analysis due to the following reasons.

(i) The Pyrosequencing results are read automatically by the instrument, and were superior in their fidelity. In PCR-RFLP analyses, the presence of partial digestion is used as a criterion for multiple allele determination. Partial digestion can occur for reasons other than the presence of multiple alleles. Improper reaction conditions including high glycerol concentration or high template DNA concentrations, among other reasons, can lead to partial digestion (Sambrook et al., 1989). For example, the undigested product in sample 3 (Fig. 3) is clearly due to some experimental artifact and not the presence of multiple alleles, as the dideoxy sequencing (Fig. 4) and Pyrosequencing (Figs. 1 and 2) assays unequivocally establish.
With dideoxy sequencing, the chromatograms generated by automated sequencers indicate the presence of multiple alleles by the appearance secondary peaks under the primary peaks at the polymorphic sites. However, such small peaks may be present in other non-polymorphic regions. In samples 5 and 9 (Fig. 3), such spurious peaks are present at the *Hha*1 site, making it difficult to assess whether or not they are genuine peaks caused by the presence of multiple alleles. Another disadvantage is the fact that the data has to be checked manually as the sequencer may assign an individual base call instead of 'N', as in the *Sau*3A1 site of sample 9 (Fig. 4). With Pyrosequencing, the assays have built in quality controls, including negative controls flanking the polymorphic locus, while the rest of the sequence acts as positive control.

(ii) It has been reported that Pyrosequencing assays can detect minor alleles down to a concentration as low as 5% (Wasson et al., 2002). In the present study, the lowest concentration used was 10% and this was

easily detected and quantified by Pyrosequencing (Fig. 1, sample 8). The same sample produced a very prominent undigested band with PCR-RFLP (Fig. 3, sample 8), with a digestion pattern similar to sample 1. Thus, it can be assumed that the undigested band was a result of improper digestion, rather than the presence of the second allele. The presence of the second allele was not detected by sequencing in sample 8 as the chromatogram at the *Sau*3A1 site showed a secondary peak, which was very small and barely discernible (Fig. 4, sample 8). Similarly, digestion of sample 7 produced very faint bands of digested products (Fig. 3, sample 7), which can be easily overlooked. Thus, the Pyrosequencing assay is more sensitive than PCR-RFLP and dideoxy sequencing in detecting low levels of multiple alleles.

(iii) The Pyrosequencing assay is rapid and less tedious than PCR-RFLP and dideoxy sequencing. A single duplex PCR cannot only determine the genotype but can also detect the presence of multiple genotypes. The accurate quantification of the alleles can be made from the actual peak height of the duplex assay. The duplex format designed here is a rapid technique for genotype detection, yielding results approximately 4 h after extraction of DNA. In contrast, for PCR-RFLP, two PCR reactions are needed, followed by restriction digestion, to determine the genotype at each of the two loci and would require a continuous bench time of 12 h. Dideoxy sequencing is even more labor intensive, even if done directly from PCR products and would require more than 15 h. Microsatellite analysis is also laborious and would require multiple PCR runs, depending on the number of loci examined, apart from the time required for the electrophoretic analysis.

(iv) Neither PCR-RFLP nor sequencing provides means to directly measure the level of the different alleles present in a mixed sample. At best, they can detect the presence of multiple alleles. In contrast, the levels of the different alleles can be estimated with reasonable accuracy using Pyrosequencing assay.

(v) Pyrosequencing assay would also be more suitable for analyzing putrefied and degraded samples. Chaturvedi et al. (1998) and Gabriel et al. (2001) demonstrated that fragments smaller than 250 bp were more consistently amplified by PCR from putrefied samples. Thus, the smaller amplicons (77 and 88 bp in this case) have better chances of getting amplified, compared with the larger amplicons (~250 bp) that are used for PCR-RFLP of *SAG2* loci. The Pyrosequencing reaction is able to read the first base following the sequencing primer.

Investigations are being carried on in numerous laboratories to understand the dynamics of *T. gondii* infection with relation to the genotype (Dubey et al., 2005 and references therein), as well as determination of multiple genotype infections. The Pyrosequencing assay reported here provides a simple and efficient means to diagnose the presence of *T. gondii* DNA, determine the genotype, and also to detect and quantify multiple genotype infections in a single assay.

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2005.03.017.

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